I, Dr. Hendrik Ehlich

confirm that I am conversant with the English and German languages and I am a competent translator from one to the other. I declare that to the best of my knowledge and belief the attached English translation is a true and correct translation of the Specification of German Patent Application DE 199 26 475.9.

H. Ehlich

Signature of the Declarant

Dated the 1st of October 2007

CARRIER-DRUG CONJUGATE

The present invention relates to carrier-drug conjugates as well as methods for their preparation and medicaments that contain the conjugates.

Many of the drugs now used are low-molecular-weight compounds and exhibit a high plasma clearance or total body clearance after systemic administration. Furthermore, they penetrate into the tissue structures of the body by diffusion processes and exhibit, as a rule, a uniform biodistribution. Both properties result in only small quantities of the drug reaching the place of action, and the drug brings about side effects on the healthy tissues of the body because of its distribution. These disadvantages are especially marked in the case of such drugs that possess a high cytotoxic potential, such as cytostatics or immunosuppressants.

New derivatives or formulations that permit a more selective therapy are therefore sought. To this end, chemoimmunoconjugates, or protein conjugates or polymer conjugates, respectively, made up of a suitable carrier substance and a drug, are being developed.

With regard to the related art in this area, mention should be made of polymer conjugates in which cytostatics are coupled to serum proteins, antibodies, growth factors, hormone-like or peptide-like structures, or synthetic polymers (Mägerstädt, M., Antibody Conjugates and Malignant Disease, Library of Congress, 1990; Seymour, L. W., *Crit. Rev. Ther. Drug Carrier Sys.* (1992), 9, 135-187; Maeda, H., and Matsumura, Y., *Crit. Rev. Ther. Drug Carrier Sys.* (1989), 6, 193-210).

DE-A-41 22 210 describes conjugates of tumor-active compounds with albumin, the tumor-active compound being activated with N-hydroxysuccinimide and carbodiimide and the mixture so obtained being coupled directly to the carrier protein. The disadvantages of these conjugates are, among other things, that they cannot be obtained in the requisite high purity, the native structure of the albumin often does not

remain intact on account of the preparation method, and the stoichiometric ratio of drug to albumin is inconstant and poorly reproducible. Furthermore, these conjugates do not allow of being released in suitable fashion in the target tissue or in the target cells.

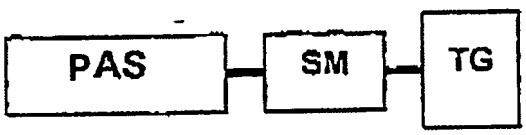
It is therefore an object of the present invention to provide new carrier-drug conjugates that overcome the disadvantages of the conjugates known heretofore.

This object is achieved through the embodiments of the present invention characterized in the Claims.

In particular, there is provided a carrier-drug conjugate comprising a carrier, which contains a polypeptide sequence having one or a plurality of cysteine groups, and a drug, which contains a pharmaceutically active substance, a spacer molecule, and a thiol-binding group, wherein more than 0.7 mol, preferably at least 0.9 mol, of drug per mol of cysteine group are being bound to the carrier via the thiol-binding group. The expression "pharmaceutically active substance" means that the substance in question brings about a pharmacological effect either by itself or after its conversion by metabolism in the organism in question, and thus also includes the derivatives resulting from these conversions. Naturally, the pharmaceutically active substance can exhibit a single (for example as a cytostatic only) or a broad pharmacological action spectrum (for example as a cytostatic and as an antiphlogistic).

According to a preferred embodiment of the conjugate according to the invention, the carrier is native or recombinant albumin.

The drug or the drug derivative in the conjugate according to the invention can be represented, for example, by the following scheme (PAS, pharmaceutically active substance; SM, spacer molecule; TG, thiol-binding group):



The conjugate according to the invention represents a transport and/or depot form of the pharmaceutically active substance, which thus reaches the target cells or the target tissue of the drug in targeted manner or in metered form. In contrast to the previously known conjugates, the conjugates of the present invention can be obtained in a higher purity, the native structure of the carrier remains intact, and the stoichiometric ratio of drug to carrier is constant and reproducible.

In contrast to the albumin-cytostatic conjugates described in DE-A-41 22 210, the conjugate according to the invention further has the advantage that a spacer molecule is present between the pharmaceutically active substance and the thiol-binding group, which spacer molecule is tailored such that the pharmaceutically active substance or a corresponding active derivative thereof can be released hydrolytically and/or in pH-dependent fashion and/or enzymatically in the target tissue or in the target cells.

Carriers such as for example albumin or its drug conjugates exhibit a markedly long half-life in the systemic circulation (up to 19 days - Peters, T., Jr. (1985): Serum Albumin, *Adv. Protein. Chem.* 37, 161-245). Because of an elevated permeability of vessel walls of the malignant, infected or inflamed tissue for macromolecules, the carrier such as for example serum albumin passes preferentially into the target tissue (Maeda, H., and Matsumura, Y., *Crit. Rev. Ther. Drug Carrier Sys.* (1989), *6,* 193-210). As a result, an active substance coupled to a carrier, for example albumin, can reach the place of action in more targeted fashion. Furthermore, the carrier-drug conjugate according to the invention prevents the pharmaceutically active substance from diffusing into healthy tissue structures of the body or from being eliminated via the kidney or injuring the kidney as much as the unbound pharmaceutically active substance. As a result, the pharmacokinetic profile of the pharmaceutically active substance is modified and improved, because the action of the pharmaceutically active substance is increased by a buildup at the place of action and, at the same time, the toxic effects on healthy systems of the body are diminished.

The conjugate of the present invention has excellent solubility in water. Furthermore,

the conjugate according to the invention shows, *in vivo,* for example, an improved antitumoral effectiveness in comparison to the unbound pharmaceutically active substance.

According to a preferred embodiment of the conjugate according to the invention, the spacer molecule and/or the linkage between the pharmaceutically active substance and the spacer molecule and/or the linkage between the thiol-binding group and the spacer molecule is cleavable in pH-dependent fashion and/or enzymatically. Preferably, the spacer molecule and/or the linkage between the pharmaceutically active substance and the spacer molecule and/or the linkage between the thiol-binding group and the spacer molecule contains at least one acid-labile bond. Examples of acid-labile bonds are ester, acetal, ketal, imine, hydrazone, carboxylhydrazone and sulfonylhydrazone bonds.

According to a further embodiment of the conjugate according to the invention, the spacer molecule and/or the linkage between the pharmaceutically active substance and the spacer molecule and/or the linkage between the thiol-binding group and the spacer molecule contains at least one peptide bond. The peptide bond preferably lies within a peptide sequence that contains at least one cleavage sequence of a protease. The at least one peptide bond can therefore be implemented by the insertion of a peptide sequence into the spacer molecule and/or into the linkage between the pharmaceutically active substance and the spacer molecule and/or into the linkage between the thiol-binding group and the spacer molecule; that is, the linkage in question is a peptide bond and is preferably made up of about 1 to 30 amino acids. The peptide sequence is thus preferably tailored to the substrate specificity of certain of the body's own enzymes or of enzymes that occur in or are formed by microorganisms. In this way, the peptide sequence or a part of this sequence is recognized in the body by the enzymes and the peptide is cleaved.

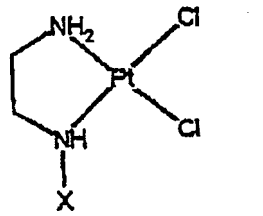
The enzymes are, for example, proteases and peptidases, for example matrix metalloproteases (MMP) or cysteine proteases, which are formed or activated in intensified manner in diseases such as rheumatoid arthritis or cancer, leading to

excessive tissue degradation, inflammations and metastasis. Target enzymes are in particular MMP 2, MMP 3 and MMP 9, which take part in the cited pathological processes as proteases (Vassalli, J., and Pepper, M. S. (1994), *Nature 370,* 14-15; Brown, P. D. (1995), *Advan. Enzyme Regul. 35,* 291-301).

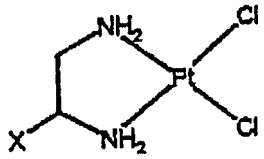
Further proteases that represent target enzymes for conjugates of the present invention are cathepsins, in particular cathepsin B and H, which have been identified as key enzymes in inflammatory and malignant diseases (T. T. Lah et al. (1998), *Biol. Chem. 379,* 125-301).

Both types of bond - acid-labile bond and peptide bond - guarantee that the pharmaceutically active substance or a correspondingly active derivative is cleaved extracellularly and/or intracellularly at the place of action and the substance can bring its pharmaceutical action into play.

According to a preferred embodiment, the pharmaceutically active substance is a cytostatic, a cytokine, an immunosuppressant, an antirheumatic, an antiphlogistic, or an antimycotic. Especially suitable cytostatics of the conjugates of the present invention are the N-nitrosoureas such as nimustine, the anthracyclines doxorubicin, daunorubicin, epirubicin, idarubicin, mitoxantrone and ametantrone as well as related derivatives; the alkylating agents chlorambucil, bendamustine, melphalan and oxazaphosphorines as well as related derivatives; the antimetabolites methotrexate, 5-fluorouracil, 2'-deoxy-5-fluorouridine and thioguanine as well as related derivatives; the taxanes paclitaxel and docetaxel as well as related derivatives; the camptothecins topotecan, irinotecan, 9-aminocamptothecin and camptothecin as well as related derivatives; the podophyllotoxin derivatives etoposide, teniposide and mitopodozide as well as related derivatives; the *Vinca* alkaloids vinblastine, vincristine, vindesine and vinorelbine as well as related derivatives; and a compound of the general formulae I to XII:



Formula I



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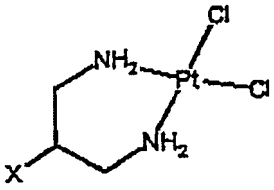
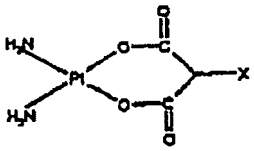


Formula III

Formula IV



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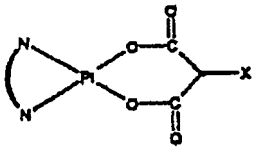

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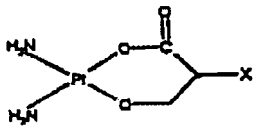

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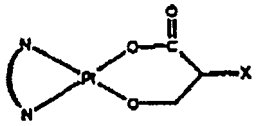

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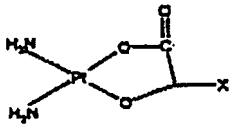
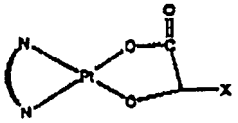
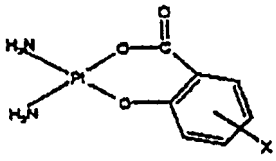


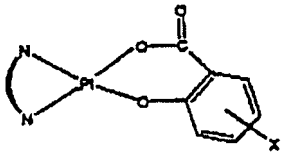
Formula X

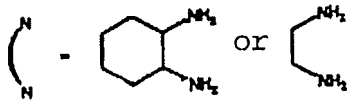


Formula XI



Formula XII





where X is the spacer molecule or the thiol-binding group.

Especially suitable cytokines in conjugates of the present invention are, for example, interleukin 2, interferon α-2a, interferon α-2b, interferon β-1a, interferon β-1b, interferon γ-1b and related derivatives. The cytokines used are, as a rule, medicaments prepared using genetic engineering.

Especially suitable immunosuppressants in conjugates of the present invention are, for example, cyclosporin A, FK 506 and related derivatives.

Especially suitable antirheumatics in conjugates of the present invention are, for example, methotrexate and related derivatives.

Especially suitable antiphlogistics in conjugates of the present invention are, for example, salicylic acid derivatives such as for example acetylsalicylic acid and related derivatives; drug derivatives having an acetic or propionic acid group such as diclofenac or, respectively, indomethacin or ibuprofen or, respectively, naproxen; and aminophenol derivatives such as for example paracetamol.

Especially suitable antimycotics in conjugates of the present invention are, for example, amphotericin B and related derivatives.

Of course, a single drug species (for example a drug with a cytostatic as pharmaceutically active substance) or various drug species (for example a plurality of distinct cytostatics or a cytostatic and an antiphlogistic, etc., as pharmaceutically active substance) can be present in combined form per mole in the conjugate according to the invention.

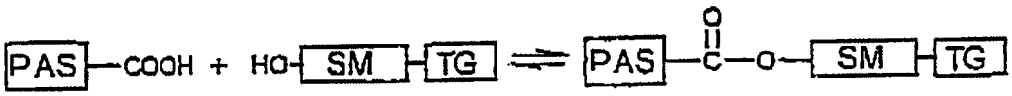
According to a further preferred embodiment of the conjugate according to the invention, the spacer molecule comprises a substituted or unsubstituted, branched-chain or straight-chain aliphatic alkyl group with 1 to 12 carbon atoms and/or at least one substituted or unsubstituted aryl group.

In a further preferred embodiment of the conjugate according to the invention, the thiol-binding group comprises a maleinimide group, a haloacetamide group, a haloacetate group, or a pyridyldithio group, which are substituted if appropriate.

The drug or drug derivative of the conjugates according to the invention can be

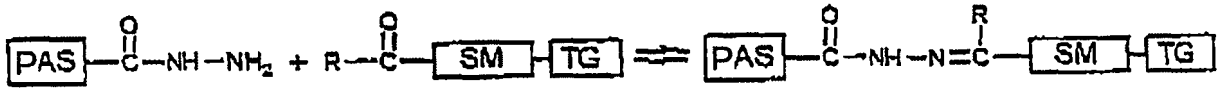
prepared according to one of the following general descriptions, depending on what functional group is present.

Drugs or drug derivatives, having an HOOC group, of the conjugates according to the invention can be derivatized, for example, in the following way:



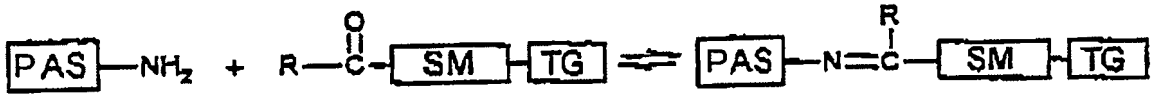
Esterification here is effected with methods known in the related art.

It is further possible to convert the HOOC group to a hydrazide group, for example by reaction with *tert*-alkylcarbazates followed by cleavage with acids (described in DE-A-196 36 889), and to react the drug, having a hydrazide group, with a group containing a carbonyl component and made up of the thiol-binding group and the spacer molecule, as is described, among others, in DE-A-196 36 889:



R = H, alkyl, phenyl, substituted phenyl

Drugs or drug derivatives, having an H_2N group, of the conjugates according to the invention can be derivatized, for example, in the following way:

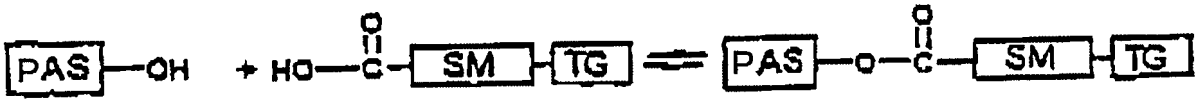


R = H, alkyl, phenyl, substituted phenyl

The reaction to the imine derivatives here is effected with methods known in the related art.

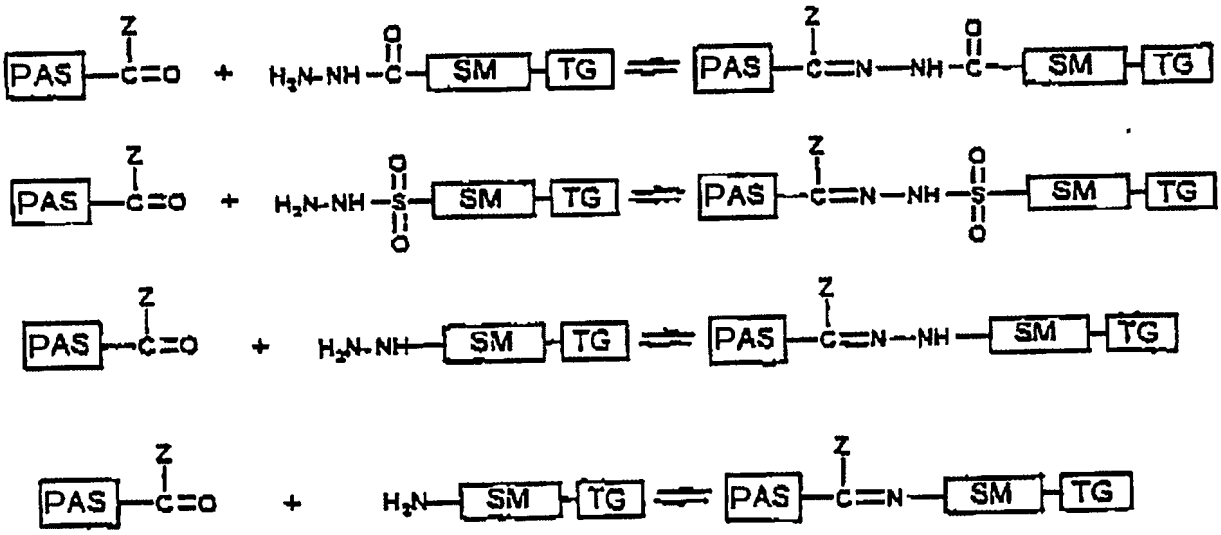
Drugs or drug derivatives, having an HO group, of the conjugates according to the

invention can be derivatized, for example, in the following way:



Esterification here is effected with methods known in the related art.

Drugs or drug derivatives, having a carbonyl component, of the conjugates according to the invention can be derivatized, for example, in the following way:



Z = chemical group of the pharmaceutically active substance

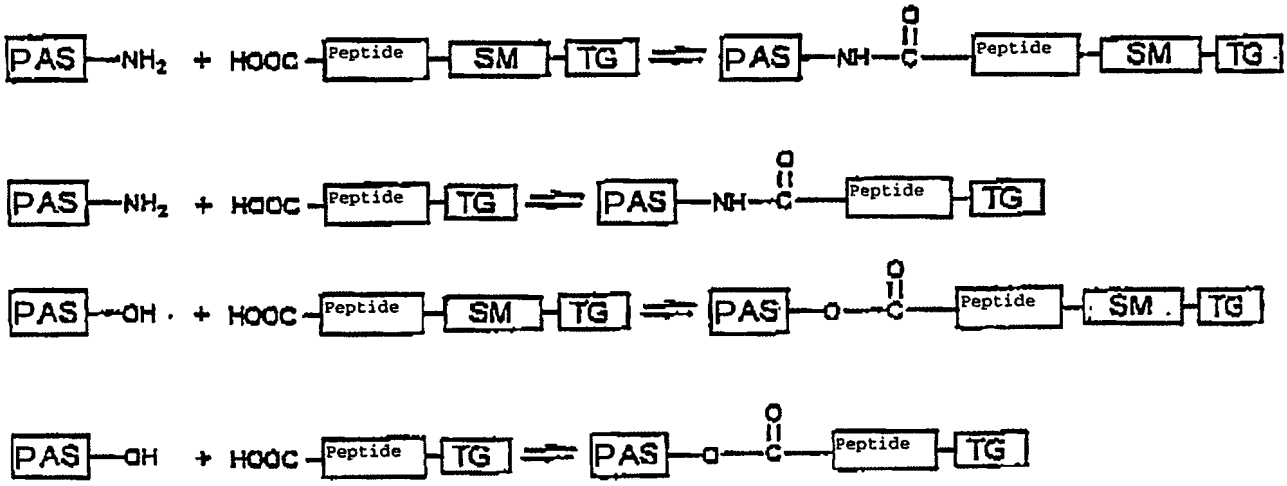
The reaction to the carboxyhydrazone, sulfonylhydrazone, hydrazone and imine derivatives here is effected with methods known in the related art.

The groups that are made up of the thiol-binding group and the spacer molecule can be prepared, for example, according to methods that are described in, among others, DE-A-196 36 889, U. Beyer et al. 1997 (*Chemical Monthly, 128,* 91, **1997**), R. S. Greenfield et al., 1990 (*Cancer Res.*, 50, 6600, **1990**), T. Kaneko et al., 1991 (*Bioconjugate Chem.*,

2, 133, **1991**), Bioconjugate Techniques (G. T. Hermanson, Academic Press, 1996), or in U.S. Patent No. 4,251,445.

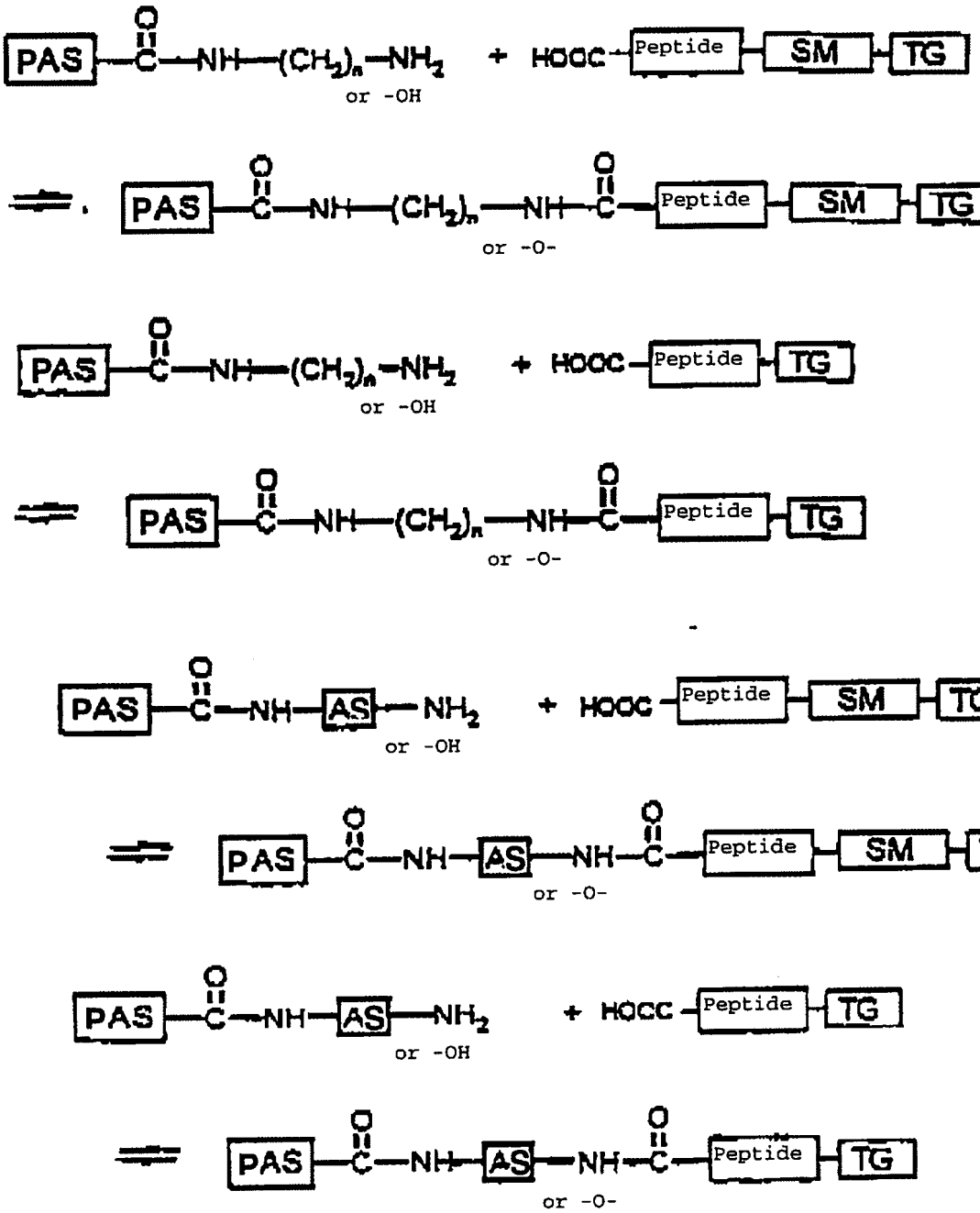
Drugs or drug derivatives, containing a peptide bond, of the conjugates according to the invention can be prepared, for example, by reacting a peptide that is made up of 2 to about 30 amino acids with a thiol-binding compound, so that a thiol-binding group is introduced directly or via a spacer molecule at the N-terminal end of the peptide.

In the presence of a condensation agent such as for example N,N'-dicyclohexylcarbodiimide (DCC) or N-cyclohexyl-N'-(2-morpholinoethyl)carbodiimide methyl-*p*-toluenesulfonate (CMC), and if appropriate with the addition of N-hydroxysuccinimide or of a water-soluble N-hydroxysuccinimide such as for example N-hydroxysuccinimide-3-sulfonic acid sodium salt, or 1-hydroxybenzotriazole, and/or in the presence of a base, for example N-methylmorpholine or triethylamine, the peptide derivatives so obtained can be reacted to the corresponding thiol-binding drug-peptide derivatives with drugs or drug derivatives that have an H_2N or HO group:



It is further possible, via the HOOC group of the drugs of the conjugates according to the invention, to introduce an H_2N or HO group, for example by derivatization via the α-amino group of the amino acids lysine, serine or threonine or with a diamino compound

of the general formula $H_2N-(CH_2)_n-NH_2$ or an alcoholamine of the general formula $H_2N-(CH_2)_n-OH$ with n = 1 to 12, and then to react these derivatives with the above-cited peptide derivatives to the corresponding thiol-binding drug-peptide derivatives:



AS = lysine, serine or threonine

The substrate specificity of target enzymes such as for example of MMP 2, MMP 3, MMP 9, cathepsin B and H is known (Netzel-Arnett et al. (1993), *Biochemistry 32,* 6427-6432, Shuja, S., Sheahan, K., and Murname, M. J. (1991), *Int. J. Cancer 49,* 341-346, Lah, T. T., and Kos, J. (1998), *Biol. Chem. 379,* 125-130).

For example, octapeptides (P_4-P'_4) for MMP 2 and MMP 9 have been identified (see Table 1), which octapeptides simulate the cleavage sequence of the collagen chain and are cleaved with particular efficiency by MMP 2 and 9 (in what follows, amino acids are abbreviated in accordance with the international three-letter code):

Table 1:

Peptide							
P_4	P_3	P_2	P_1	P'_1	P'_2	P'_3	P'_4
Gly	Pro	Leu	Gly	Ile	Ala	Gly	Gln
Gly	Pro	Gln	Gly	Ile	Trp	Gly	Gln

(Netzel-Arnett et al., *Biochemistry 32,* **1993**, 6427-6432)

The peptides are enzymatically cleaved exclusively at the P_1-P'_1 bond.

Furthermore, in the case of cathepsin B, substrate-specific peptides are known with the sequence -Arg-Arg- or -Phe-Lys- (Werle, B., Ebert, E., Klein, W., and Spiess, E. (1995), *Biol. Chem. Hoppe-Seyler 376,* 157-164; Ulricht, B., Spiess, E., Schwartz-Albiez, R., and Ebert, W. (1995), *Biol. Chem. Hoppe-Seyler 376,* 404-414).

The peptide sequence that contains intended peptide cleavage points relevant for the target enzyme can also be constructed such that the intended peptide cleavage point is repeated a plurality of times, for example by:

Gly-Pro-Leu-Gly—Ile-Ala-Gly-Gln-Gly-Pro-Leu-Gly—Ile-Ala-Gly-Gln

or

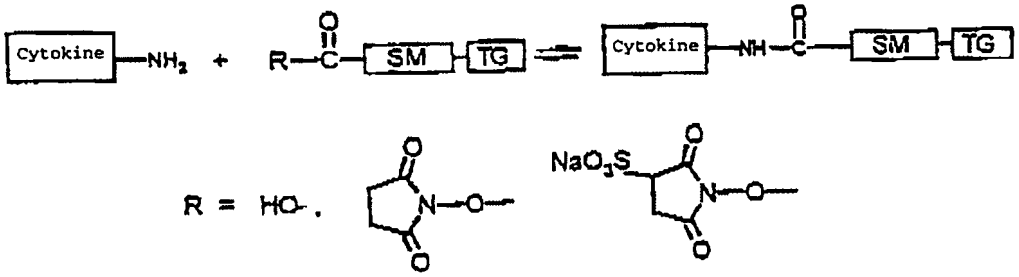
Phe-Lys-Phe-Lys-Phe-Lys-Phe-Lys-Phe-Lys-Phe-Lys-

or a repetitive peptide sequence can be integrated that increases the distance between the thiol-binding group and the relevant intended peptide cleavage point, as for example by:

-$(Gly)_n$-Phe-Lys-Phe-Lys-

with, preferably, n = 2 to 20, more preferably $n \leq 12$.

An important feature of this embodiment of the conjugate according to the invention is that the intended peptide cleavage point relevant for the target enzyme in question is present at least once in an oligopeptide made up of roughly 1 to 30 amino acids. The above-cited oligopeptides are representative examples for the enzymatically cleavable bond in the conjugates according to the invention and do not restrict the invention.



Drugs or drug derivatives, containing a cytokine, of the conjugates according to the invention can be prepared, for example, by reacting the cytokine with a spacer molecule containing a thiol-binding group, which spacer molecule exhibits a carboxylic acid or an activated carboxylic acid:

If the spacer molecule exhibits an N-hydroxysuccinimide ester group (N-hydroxysuccinimide or N-hydroxysuccinimide-3-sulfonic acid sodium salt), it is reacted directly with the cytokine. The reaction of the cytokine with a spacer molecule containing a thiol-binding group, which spacer molecule exhibits a carboxylic acid, to the corresponding thiol-binding derivatives takes place in the presence of a condensation agent, such as for example N,N'-dicyclohexylcarbodiimide (DCC) or N-cyclohexyl-N'-(2-morpholinoethyl)-carbodiimide methyl-*p*-toluene sulfonate (CMC), and if appropriate with the addition of N-hydroxysuccinimide or N-hydroxysuccinimide-3-sulfonic acid sodium salt. As a rule, the cytokines derivatized in this way are purified with the aid of exclusion chromatography. The above-described reactions are well known to a person skilled in the art (see, e.g., Bioconjugate Techniques, G. T. Hermanson, Academic Press, 1996).

The above-described drugs or drug derivatives are coupled to a carrier containing a polypeptide sequence with one or a plurality of cysteine groups, such as for example native or recombinant albumin, so that more than 0.7 mol, preferably at least 0.9 mol, of drug per mol of cysteine group is bound to the carrier via the thiol-binding group. If the polypeptide sequence of the carrier contains n (for example 3) cysteine groups, this means that 1 mol of this carrier contains n (for example 3) mol of cysteine groups, and thus a maximum of n (for example 3) mol of drug can be present bound to the carrier per mol of the corresponding conjugate. In the conjugate according to the invention, therefore, 100% of the cysteine groups present in the carrier are ideally bound with a drug via the thiol-binding group.

A further embodiment of the present invention thus relates to a method for the preparation of a conjugate as defined above, comprising

(i) treatment of the carrier with a reducing agent so that more than 0.7 mol, preferably at least 0.9 mol, of cysteine SH groups is present in the carrier per mol of cysteine group and
(ii) coupling of the drug to the cysteine SH groups in the carrier via the thiol-

binding group.

In a preferred embodiment of the method according to the invention, the reducing agent used for the treatment of the carrier is dithiothreitol (DTT), dithioerythritol (DTE) or mercaptoethanol. The especially preferred reducing agent is DTT.

The method according to the invention is based on the knowledge that the carriers known in the related art exist in an inhomogeneous oxidation state. For example, in the case of commercially available native albumin, as a rule, the Ellmann photometric assay detects ≈ 0.2 to 0.7 mol of HS groups per mol of cysteine groups in the albumin; that is, the cysteine-34 is often oxidized by sulfur-containing compounds such as for example cysteine or glutathione via a disulfide bond. This means that the cysteine SH groups present in the albumin are at least often not free, which formerly led to the condition that the yield of prepared conjugates was too low and/or strongly fluctuating from one albumin charge to another albumin charge.

It has been established according to the invention that commercially available carriers can be treated with a reducing agent, the cysteine groups oxidized via disulfide bonds being reduced so that more than 0.7 mol of cysteine SH groups is present per mol of cysteine groups in the carrier. The reaction is preferably controlled such that at least 0.9 mol of cysteine SH groups becomes available per mol of cysteine group in the carrier.

The reaction of the reducing agent with a commercially available carrier, for example albumin, takes place, for example, in a salt buffer, for example in 0.01 M sodium borate, 0.15 M NaCl, 0.001 M EDTA or 0.15 M NaCl, 0.004 M phosphate in a pH range of 5.0 to 8.0, preferably 6.0 to 7.0. The reducing agent can be inlet in excess; preferably the ratio of reducing agent to carrier is between 0.5:1 and 10:1. The reaction time is between 1 h and 96 h, preferably between 6 h and 24 h.

The carrier treated with the reducing agent is isolated, for example, by gel filtration (for example Sephadex® G10 or G25; solvent 0.004 M phosphate, 0.15 M NaCl, pH 7.4) or

by ultrafiltration.

The concentration of carrier after gel filtration has been carried out is determined using the extinction coefficient at 280 nm; the number of HS groups inserted is determined with Ellmann's reagent at 412 nm. The carrier solution thus isolated can be used directly for the synthesis of the conjugates. It is also possible to concentrate the carrier solution with a commercially available concentrator or to lyophilize it. The isolated carrier solution or the lyophilizate can be stored in the temperature range of -78 to +30 °C.

The coupling of the above-described drug derivatives to the carrier takes place, for example, at room temperature. To the carrier, which is in a salt buffer (for example 0.15 M NaCl, pH 6.0 to 8.0), which was previously degassed if appropriate, there is added a roughly 1.1-fold to 10-fold excess of the drug prepared as described above (relative to the number of HS groups present in the carrier), dissolved in a minimal quantity of solvent, for example DMF, dimethyl sulfoxide, water, salt buffer, ethanol, methanol, propylene glycol, glycerin, acetonitrile or THF (roughly 1 to 10% of the volume of the test portion of carrier). It is also possible to add the drug to the carrier solution as a solid. Furthermore, it may be advantageous to add an auxiliary agent, such as for example a fatty acid or a tryptophonate derivative, to the carrier solution. After a reaction time between 5 min and 48 h, the solution is centrifuged if necessary, and the carrier-drug conjugate formed is isolated by subsequent gel filtration (for example Sephadex® G10 or G25) in a salt buffer, such as for example 0.004 M phosphate, 0.15 M NaCl, pH 6.0 to 8.0.

The purity of the conjugate obtained can be verified, for example, by HPLC, for example by gel chromatography. In contrast to commercially available conjugates, the conjugates prepared according to a preferred embodiment of the method according to the invention exhibit a purity of more than 95%.

The solution of the conjugate so obtained can be concentrated with a commercially available concentrator. The conjugates can be stored in dissolved form at +1 to +30 °C

or in frozen form at T = 0 °C to -78 °C. It is further possible to lyophilize the solution of the conjugates and to store the lyophilizate at +30 ° to -78 °C.

A further embodiment of the present invention relates to a medicament containing a conjugate as defined above and, if appropriate, a pharmaceutically compatible carrier and/or auxiliary agent and/or a diluting agent. The medicament according to the invention can preferably be used for the treatment of cancer diseases, autoimmune diseases, acute or chronically inflammatory diseases, and diseases that are caused by viruses or microorganisms such as for example bacteria and/or fungi.

The figures show:

Fig. 1 (A) is an HPLC chromatogram of a conjugate according to the invention (A-DOXO-HYD-C). The plot shows the absorption at 495 nm versus the retention time in min. (B) is the corresponding HPLC chromatogram of commercially available native albumin (Immuno GmbH).

Fig. 2 shows the graphical representation of the weights and volumes of kidneys and renal tumors (A) as well as the lung weights and the number of pulmonary metastases (B) of mice in which a renal carcinoma was induced, and that were subjected to the cited treatments (control: no treatment; albumin control: native albumin; Doxo: doxorubicin; A-DOXO-HYD-C: conjugate according to the invention). For comparison, the data for mice not injected with tumor cells are also shown (no tumor).

The following example explains the present invention in greater detail without restricting it.

Example

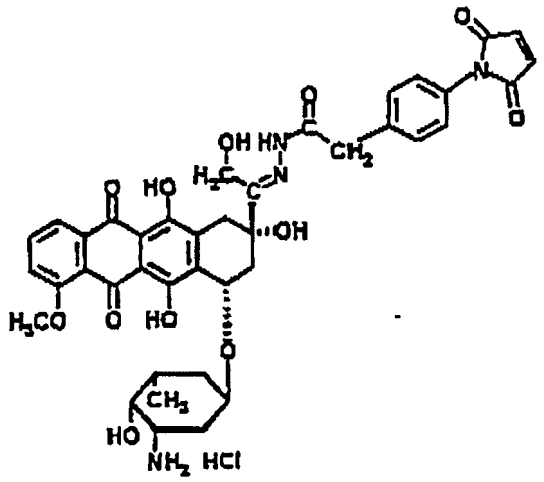
Reaction of Human Serum Albumin (HSA) with Dithiothreitol (DTT)

The method for the treatment of HSA with a reducing agent is illustrated more exactly by the following example: 2.0 g of human serum albumin (10 mL of a 20% HSA solution, Pharma Dessau) is diluted with 10 mL of buffer A (0.004 M sodium phosphate, 0.15 M NaCl, pH 7.0) and added to 100 µL of a freshly prepared 0.036×10^{-2} M solution of DTT (5.55 mg of DTT dissolved in 100 µL of buffer A), and the reaction vessel, is shaken gently for 16 h at room temperature. The albumin solution is then purified by gel filtration (5.0 cm x 25.0 cm column, Sephadex® G25; solvent buffer 0.004 M sodium phosphate, 0.15 M NaCl, pH 7.4). The protein concentration after gel filtration was determined by photometry at 280 nm ($\varepsilon(HSA)_{280}$ = 35,700 M^{-1} cm^{-1}, c[HSA] ≈ 3.1×10^{-4} M), and the number of HS groups introduced was determined with Ellmann's reagent at 412 nm (ε_{412} = 13,600 M^{-1} cm^{-1}, c[HS groups] ≈ 3.07×10^{-4} M). In the HSA thus treated, there is accordingly 0.99 mol of free cysteine SH groups per mol of cysteine group. The treated HSA was concentrated to roughly 1.0×10^{-3} M (Centriprep-10®) and used directly for the coupling reaction, described in what follows, with a thiol-binding drug of the present invention.

Preparation of the Conjugate A-DOXO-HYD-C according to the Invention

The HSA-doxorubicin conjugate (A-DOXO-HYD-C), made up of HSA treated with DTT in accordance with the above example, and a maleinimidophenylacetic acid hydrazone derivative of doxorubicin (DOXO-HYD), was further prepared in the following way.

Structure of DOXO-HYD:

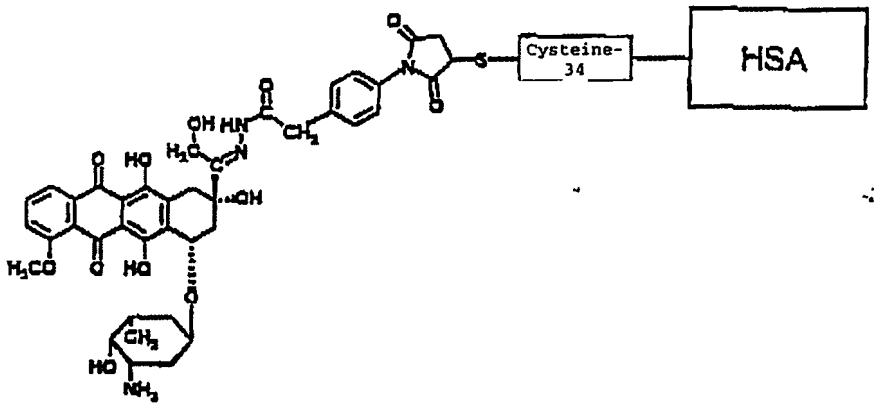


12 mL of the HSA test portion treated with DTT (sulfhydryl content of 0.99 mol per mol of HSA) was added to 0.6 mL of a solution of DOXO-HYD (Mr 807.8) in DMF (12.5 mg dissolved in 0.6 mL of DMF), and the reaction solution, sealed, was shaken for 18 h. The product HSA-doxorubicin conjugate was isolated using a Sephadex® G-25F column (column 5.0 cm x 25 cm) (retention volume 85-135 mL). The quantity of bound doxorubicin was determined with the help of the extinction coefficient of doxorubicin at 495 nm (ε_{495} = 10,650 M^{-1} cm^{-1} at pH 7.4). According to the determination, 0.97 mol of doxorubicin per mol of cysteine group in the HSA is bound to the HSA in this example.

Methods: FPLC for the preparation of the conjugates: P-500 pump, LCC 501 controller (Pharmacia) and LKB 2151 UV monitor. The protein concentration of the conjugate was determined photometrically and also by the BCA protein assay (Pierce, U.S.A.).

The purity of the A-DOXO-HYD-C conjugate was checked by HPLC with the aid of an analytical column (Bio-Sil SEC 250 (300 mm x 7.8 mm), Bio-RAD (mobile phase, as a rule, 0.15 M NaCl, 0.01 M NaH_2PO_4, 5% CH_3CN, pH 7.0) at λ = 495 nm. The HPLC chromatograms for A-DOXO-HYD-C and of commercially available native albumin (Immuno GmbH) are presented in Fig. 1A (A-DOXO-HYD-C) and Fig. 1B (native albumin). It can be clearly seen that A-DOXO-HYD-C exhibits an excellent purity, comparable with the commercially available native albumin.

Structure of A-DOXO-HYD-C:



(HSA = human serum albumin)

Biological examination

As an example for the *in vivo* effectiveness of the conjugates according to the invention, the biological data of the HSA-doxorubicin conjugate A-DOXO-HYD-C are presented.

In the "RENCA" (renal cell carcinoma) model, doxorubicin and the conjugate A-DOXO-HYD-C according to the invention were compared with each other with respect to antitumoral action at approximately equitoxic dose (intravenous therapy, 10 days after injection of roughly 1 million renal carcinoma cells into the left kidney).

Animals: Balb/c mice, female; tumor: RENCA, renal cell carcinoma; therapy: day (d) 10, 14, 18, 21 intravenous (i.v.); end of trial: d 25.

The results of these studies are summarized in Table 2.

Table 2

Number of mice	Substance	Dose (mg/kg/inj.)	Mortality (d)	Average loss of body weight (%), d 1 to 25
10	Control		2	-14
10	Albumin control	4 x 1.4 g	1	-16
10	Doxorubicin (doxo)	4 x 6 mg/kg	1	-21
10	A-DOXO-HYD-C	4 x 12 mg/kg	0	-18

The dose is relative to the quantity of doxorubicin present. The dosages of doxorubicin and A-DOXO-HYD-C are approximately equitoxic (see loss of body weight in Table 2).

The results of this experiment are, further, graphically illustrated in Fig. 2 with respect to the weights and volumes of the kidneys and renal tumors (Fig. 2A) and the lung weights and the number of pulmonary metastases (Fig. 2B). A-DOXO-HYD-C shows a very good antitumoral effectiveness and brings about a complete remission in all animals. Macroscopically visible pulmonary metastases could be observed in only one animal (Fig. 2B). In the group treated with doxorubicin, plainly visible renal tumors were observed in all animals (Fig. 2A); that is, in contrast, no complete remissions were brought about at the optimal dose of doxorubicin (body weight loss -21% (d 1 to 25); 1 animal died). Furthermore, the number of pulmonary metastases was on average some 100 metastases per mouse in the mice treated with free doxorubicin (Fig. 2B).

Claims

1. Carrier-drug conjugate comprising a carrier, which contains a polypeptide sequence having one or a plurality of cysteine groups, and a drug, which contains a pharmaceutically active substance, a spacer molecule, and a thiol-binding group, more than 0.7 mol of drug per mol of cysteine group being bound to the carrier via the thiol-binding group.

2. Conjugate according to Claim 1, in which the carrier is native or recombinant albumin.

3. Conjugate according to Claim 1 or 2, in which the spacer molecule and/or the linkage between the pharmaceutically active substance and the spacer molecule and/or the linkage between the thiol-binding group and the spacer molecule is cleavable in pH-dependent fashion and/or enzymatically.

4. Conjugate according to Claim 3, in which the spacer molecule and/or the linkage contains at least one peptide bond.

5. Conjugate according to Claim 4, in which the peptide bond lies within a peptide sequence that contains at least one cleavage sequence of a protease.

6. Conjugate according to one of Claims 3 to 5, in which the spacer molecule and/or the linkage contains at least one acid-labile bond.

7. Conjugate according to one of Claims 1 to 6, in which the pharmaceutically active substance is a cytostatic, a cytokine, an immunosuppressant, an antirheumatic, an antiphlogistic, or an antimycotic.

8. Conjugate according to Claim 7, in which the cytostatic is selected from the group of the anthracyclines, the N-nitrosoureas, the alkylating agents, the purine

antagonists or pyrimidine antagonists, the folic acid antagonists, the taxanes, the camptothecins, the podophyllotoxin derivatives, the *Vinca* alkaloids, or the *cis*-configured platinum(II) complexes.

9. Conjugate according to one of Claims 1 to 8, in which the thiol-binding group contains a maleinimide group, a haloacetamide group, a haloacetate group, or a pyridyldithio group, which are substituted if appropriate.

10. Conjugate according to one of Claims 1 to 9, in which the spacer molecule comprises a substituted or unsubstituted, branched-chain or straight-chain aliphatic alkyl group with 1 to 12 carbon atoms and/or at least one substituted or unsubstituted aryl.

11. Method for the preparation of the conjugate according to one of Claims 1 to 10, comprising
    (i) treatment of the carrier with a reducing agent so that more than 0.7 mol, preferably at least 0.9 mol, of cysteine SH groups is present in the carrier per mol of cysteine group and
    (ii) coupling of the drug to the cysteine SH groups in the carrier via the thiol-binding group.

12. Method according to Claim 11, in which the reducing agent is dithiothreitol, dithioerythritol or mercaptoethanol.

13. Method according to Claim 11 or 12, in which the conjugate prepared exhibits a purity of more than 95%.

14. Medicament containing the conjugate according to one of Claims 1 to 10 and, if appropriate, a pharmaceutically compatible carrier and/or an auxiliary agent and/or a diluting agent.

15. Medicament according to Claim 14 for the treatment of cancer diseases, autoimmune diseases, acute or chronically inflammatory diseases and diseases that are caused by viruses and/or microorganisms.

Fig. 1A

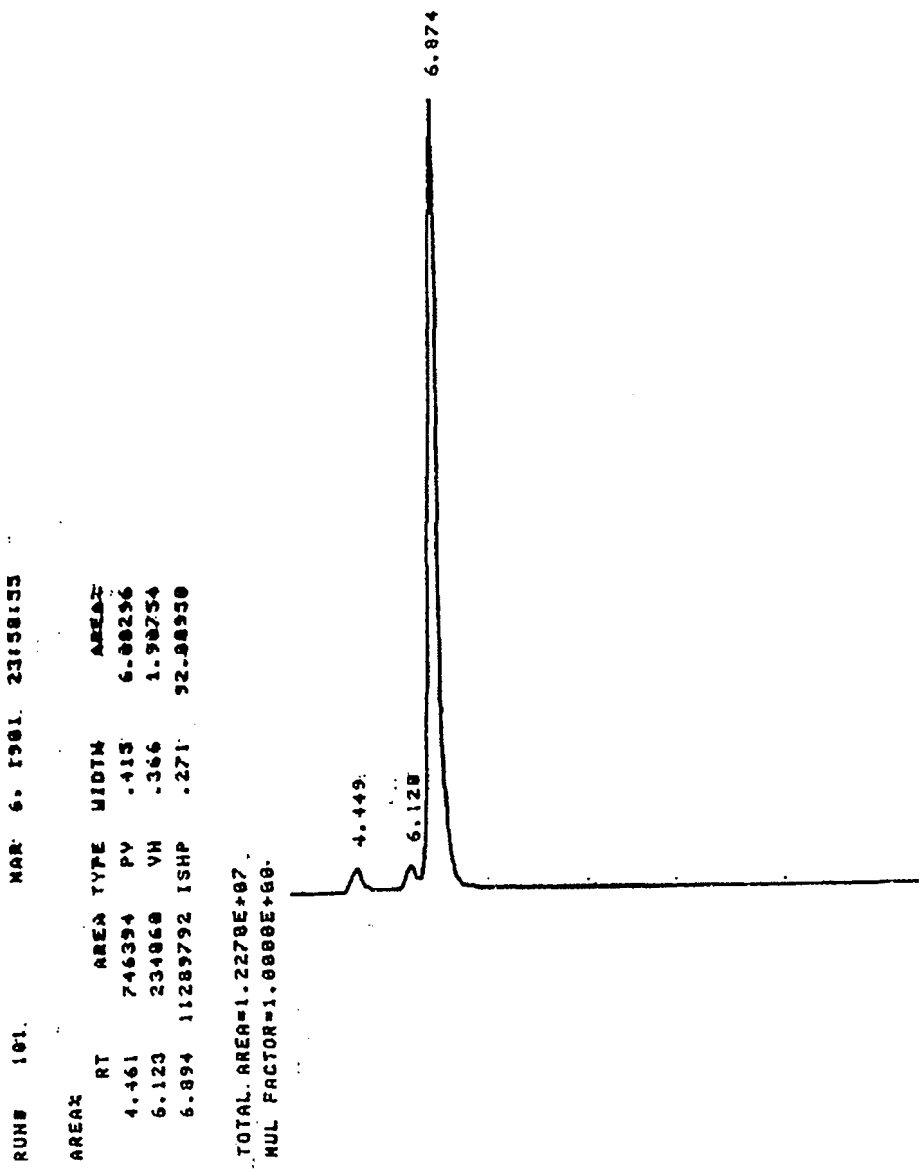

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Fig. 1B

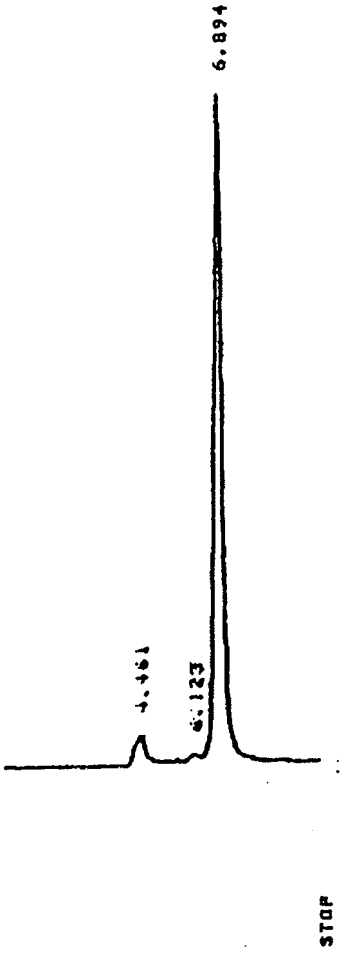
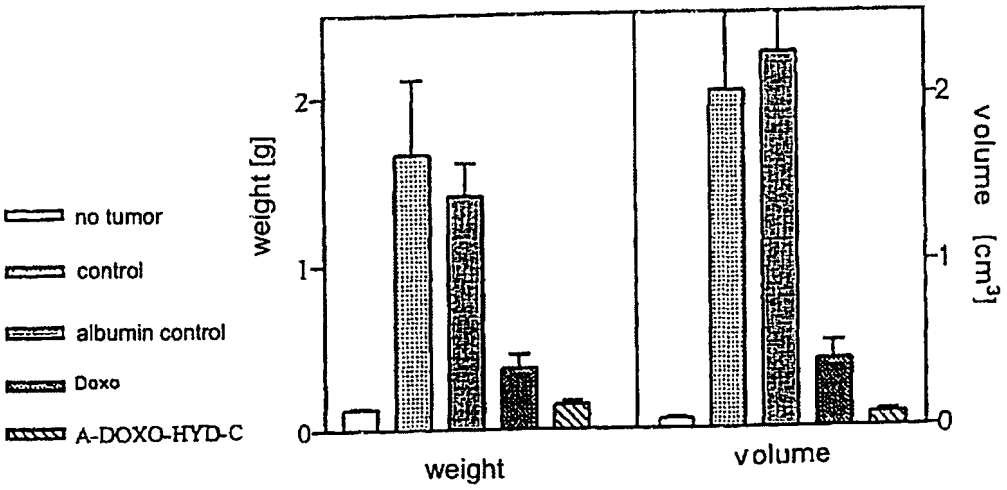


Fig. 2A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albumin control
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weight
volume
2
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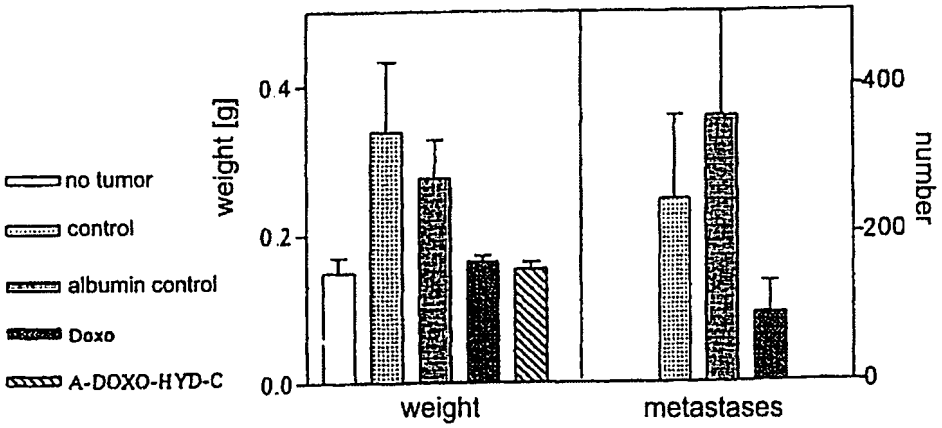
volume [cm³]

Fig. 2B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number